ASSAY OF MEMBRANE COMPLEMENT RECEPTORS (CR1 AND CR2) WITH C3b- AND C3d-COADED FLUORESCENT MICROSPHERES

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A sensitive and specific fluorescence assay for membrane complement (C) receptors (CR1 and CR2) was developed with purified C3b and C3d fragments coupled to fluorescent microspheres (0.9 μm diameter). C3-microspheres (C3-ms) bound to cells with low numbers of receptors that were undetectable by other assay techniques. Inhibition studies with anti-CR1 and anti-CR2 demonstrated that C3b-ms and C3d-ms bound exclusively to CR1 and CR2, respectively. Preparation of the C3-ms required only small amounts of partially purified C3 and no immunoglobulin or other C components. Once formed, the C3-ms were stable for up to 4 mo at 4°C.

In this report a new method for assay of CR1 and CR2 is described that requires only partially purified C3, and less of this C3 is required than for other C receptor assays. The assay is more sensitive than other assays to cells with low numbers of C receptors per cell, and the indicator C3b- and C3d-coated fluorescent microspheres are stable to storage at 4°C for up to 4 mo.

MATERIALS AND METHODS

C receptor cells. Human E, peripheral blood lymphocytes, and neutrophils were isolated as described previously (3, 8). Monocytes were removed from lymphocyte preparations by adsorption onto Sephadex G-10 (9) or isolated from lymphocyte preparations on Percoll gradients (10). The Burkitt's lymphoma-derived lymphoblastoid cell lines known as Raji and Daudi, and the BF lymphoblastoid cell line derived from transformed normal cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. Each cell type was washed two times in phosphate-buffered saline (PBS) and then suspended at 4 x 10^6/ml in 35 mM Veronal buffer, pH 7.2, containing 1% bovine serum albumin (BSA), 3.3% dextrose, 20 mM ethylene dianine tetracacetate (EDTA), and 0.2% sodium azide. The cell type was washed once again and resuspended in BDVEA to remove spontaneously released endogenous factor I (C3b-inactivator) and any other proteases that might convert the C3b-ms (see Abbreviations) to C3d-ms or C3d-ms. Also, for CR assay or neutrophils, both the C3b-ms and neutrophils were resuspended in BDVEA containing 1 mg/ml of soybean trypsin inhibitor to prevent neutrophil elastase cleavage of the C3b-ms.

C3b and C3d fragments. Human C3 was partially purified by DEAE-Sephalc (Pharmacia Fine Chemicals, Piscataway, NJ) column chromatography of the plasminogen-depleted, 5% polyethylene glycol supernatant of fresh plasma (11). C3 was cleaved to C3b with trypsin in the presence of a 50% suspension of Activated-Thiol Sepharose (ATS, Pharmacia Fine Chemicals), generating disulfide-linked C3b-Sepharose as described by Tack et al. (12). A ratio of 6 mg of C3 to 1 ml of ATS in 1 ml of 10 mM EDTA/phosphate-buffered saline, pH 7.5 (EDTA-PBS), was treated with a 0.24% weight ratio of trypsin to C3 for 15 min at 37°C with stirring. Trypsin was then inhibited by addition of a three-fold molar excess of soybean trypsin inhibitor, and the C3b-ATS was washed by centrifugation three times with 0.1 M phosphate buffer, pH 7.5, containing 0.5 NaCl and 0.1% sodium deoxycholate and three times with PBS. Elution of the C3b-ATS with 10 mM L-cysteine (raised to pH 7.0 with NaOH) demonstrated 6 mg of bound protein per milliliter of gel, all of which had the α- and β-chain structure of C3b (13) when analyzed by SDS-PAGE (14). A portion of the C3b-ATS was converted to C3d-ATS by treatment with trypsin at a 6.7:1 C3b protein to enzyme ratio for 12 hr at room temperature, followed by the same amount of trypsin for 5 more hours (12). After two washes with ice-cold PBS, elution of a sample of the C3d-ATS with 10 mM cysteine, pH 7.0, analysis by SDS-PAGE demonstrated only the 30,000 M, C3d fragment. The C3d-ATS and C3d-ATS were then eluted with 10 mM cysteine, pH 7.0, for 30 min at 20°C, and the eluted C3b and C3d were dialyzed extensively against PBS. After concentration to 2 to 3 mg/ml with a YM-10 membrane (Amicon Corp., Lexington, MA), the pure C3 fragments were stored at 0°C to 4°C with 0.02% sodium azide for up to 6 mo before use.

Antibodies to C receptors. Anti-CR1 and anti-CR2 were prepared in rabbits by immunization with purified CR1 and CR2, and used as Fab1 fragments (3, 4).

Preparation of C3b-ms and C3d-ms. Three hundred microliters of a 14% suspension of coumarin (green) or rhodamine (red) fluorescent microspheres (Covaspheres, Covalent Technology Corp., Redwood City, CA) in PBS were mixed with 100 ml of C3b or C3d (400 μg/ml in PBS) and incubated at 25°C for 1 hr on a tube rotator to allow the microspheres in suspension. The C3b-ms and C3d-ms were then pelleted and washed three times with 1% BSA/PBS by centrifugation for 10 min at 8000 x G in a Beckman Microfuge (Spinco Division of Beckman Instruments, Palo Alto, CA 94304). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
CA). The BSA in the washes effectively neutralized any remaining covalent binding sites on the microspheres. The C3b-ms and C3d-ms were then resuspended in 1.5 ml of 1% BSA/PBS containing 1.0 mM phenylmethylsulfonyl fluoride (PMSF) and sonicated briefly until a single particle suspension was obtained. A stock solution of 115 mM PMSF was first prepared by solubilizing PMSF at 20 mg/ml in 2-propanol.

C receptor assays: C receptors were assayed in parallel by: 1) rosette formation with EC3b or EC3d in BDVEA (5, 15); 2) direct immunofluorescence with fluorescein isothiocyanate-(FITC) F(ab′)-anti-CR, or anti-CR, (3, 4); 3) rosette formation with fluorescent C3b-ms or C3d-ms. One hundred microliters of a 0.14% suspension of C3b-ms or C3d-ms in BDVEA was mixed with 100 μl of C-receptor cells at 4 × 10^6/ml in a 10 × 75 mm plastic tube and then placed on a tube rotator with horizontal axis for 15 min. Unbound C3-ms were separated from the cells by layering the 200-μl cell mixture onto 5.0 ml of 6% BSA/PBS in a 12 × 75 mm plastic tube and centrifuging at 200 × G for 5 min. After aspiration of the supernatant, the pelleted cells were resuspended in residual wash fluid (≈25 μl) by shaking the tube gently and examined for bound C3-ms by fluorescence microscopy. Alternatively, with cells having a low density of C receptors (i.e., human E), C3-ms were centrifuged together with the cells at 1000 × G for 5 min and incubated as a pellet for 5 min at 37°C. After gentle resuspension of the cell pellet, unbound C3-ms were removed and the cells were examined as above. Human E that bound more than three C3-ms per cell were considered positive, whereas a positive cut-off of five or more bound C3-ms per cell was used with lymphocytes, neutrophils, monocytes, and lymphoblasts. With each cell type, nonspecific binding of microspheres was assessed with BSA-coated microspheres. Because the fluorescence of the C3-ms was so bright, it was possible to count rosettes with simultaneous fluorescence and minimal tungsten light phase contrast illumination.

Assay for C-receptor specificity of C3-ms: A pellet of 4 × 10^6 C-receptor cells was resuspended in 100 μl of F(ab′)-anti-CR, or anti-CR, at 1.0 mg/ml in BDVEA and incubated at 22°C for 15 min. Next, after two washes in BDVEA, the anti-C-receptor-treated cells were resuspended in 100 μl of BDVEA and tested for rosette formation with 100 μl of C3b-ms or C3d-ms.

Assay for binding of C3-ms to B cells and T cells. Before addition of C3-ms-tetramethylrhodamine isothiocyanate-(TRITC) B cells were stained with F(ab′)-anti-Ig-FITC (16), or T cells were stained with anti-Leu 1-FITC (Becton-Dickenson, Sunnyvale, CA). Red C3-ms rosetted cells were then simultaneously evaluated for green fluorescence surface staining. Because the red fluorescence from the TRITC-C3-ms was easily visualized and distinguishable from FITC surface staining using fluorescein-optimized illumination, it was unnecessary to switch back and forth between fluorescein and rhodamine optics for double stain assays. On the other hand, the green fluorescence with the coumarin-C3-ms rosetted cells was occasionally too bright to visualize TRITC-anti-lg surface staining using rhodamine-optimized illumination.

RESULTS

Assay of C receptors with C3b-ms and C3d-ms. CR1 and CR2 on lymphocytes and other cell types were assayed in parallel with C3-ms, EC3, and anti-CR-FITC (Table I and Figs. 1–5). In all cases, C3-ms detected the proportion of C-receptor-bearing cells than the other reagents did. In particular, with human E known to have only 900 to 2000 CR1 per cell (3, 17, 18), C3b-ms bound to nearly all of the cells, whereas EC3b bound to only 75% of human E, and anti-CR1 fluorescence was undetectable. Most human E bound more than five C3b-ms particles, and many E bound more than 20 C3b-ms per cell (Fig. 1). Likewise, with Daudi cells that are known to express low numbers of CR1 per cell, C3d-ms bound to 95% of Daudi cells, whereas EC3d bound to 86% of Daudi cells, and anti-CR1 fluorescence was detectable on only 75% of Daudi cells. With cells having low numbers of C-receptors per cell, enhanced binding of C3-ms was obtained by centrifuging the C3-ms together with the cells and then resuspending

![Figure 1](image1.png) Human E rosettes with coumarin (green)-C3b-ms. In Figures 1 to 5, fluorescein-optimized Ploem fluorescence illumination was used simultaneously with minimal tungsten light phase-contrast illumination.

![Figure 2](image2.png) B cell rosette with coumarin-C3b-ms. The unrosetted lymphocyte is probably a T cell.

![Figure 3](image3.png) Monocyte rosette with coumarin-C3b-ms. The unrosetted cells are probably T lymphocytes.

### Table I

<table>
<thead>
<tr>
<th>C-Receptor Cell Type</th>
<th>CR1 Assays</th>
<th>CR2 Assays</th>
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<tbody>
<tr>
<td></td>
<td>C3b</td>
<td>Anti-CR1</td>
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<tr>
<td>Human E</td>
<td>75</td>
<td>0</td>
</tr>
<tr>
<td>Blood lymphocytes</td>
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<td>Blood neutrophils</td>
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<td>80</td>
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<td>Lymphoblastoid lines</td>
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<td>0</td>
</tr>
<tr>
<td>Raj</td>
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<td>90</td>
</tr>
<tr>
<td>Daudi</td>
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the pellet gently. However, with monocytes and neutrophils, this technique resulted in occasional clumps of cells with C3b-ms that were difficult to enumerate.

Specificity of C3-ms. C3b-ms bound only to cells known to bear CR1, and in particular did not bind to Raji cells or T cells known to lack CR1 (15, 16). It was essential to perform C3b-ms assays with freshly washed leukocytes in an EDTA and azide-containing buffer (BDVEA) to inhibit release of endogenous factor H (H, β1H) and I, and thus prevent conversion of C3b-ms to iC3b-ms (15, 19, 20). With neutrophils, 1.0 mg/ml of soybean trypsin inhibitor was also included in the BDVEA assay buffer to inhibit neutrophil elastase activity (2). C3d-ms bound only to lymphocytes bearing CR2, and binding of C3d-ms to monocytes and neutrophils was usually not detected. Occasional nonspecific binding of C3d-ms to monocytes paralleled the nonspecific binding observed with BSA-coated microspheres, and was usually eliminated by centrifugation of rosetted cells through 6% BSA/PBS as described that removed loosely bound and unbound microspheres. When nonspecific binding of BSA-ms was observed on occasional cells (<5%), it usually amounted to only one to three BSA-ms per cell. For this reason, cells were not considered to be rosetted unless they bound five or more C3-ms.

The specificity of C3b-ms and C3d-ms for CR1 and CR2 was further confirmed by blocking experiments with anti-CR1 and anti-CR2. In all cases, anti-CR1 totally inhibited C3b-ms binding, whereas anti-CR2 totally inhibited C3d-ms binding (Table II).

Stability of C3-ms. C3b-ms stored for 3 wk at 4°C showed no loss in rosetting activity with human E. After 4 wk storage; however, C3b-ms activity with human E was reduced to 60 to 70%, whereas monocyte and lymphocyte rosetting with C3b-ms was undiminished. By contrast, C3d-ms stored for 4 mo at 4°C still bound to 95% of Daudi lymphoblasts.

Mechanism of C3 attachments to microspheres. According to the manufacturer, fluorescent microspheres bind proteins covalently by way of either amino or sulfhydral groups. Because both C3d and the d region of C3b contain a single free sulfhydral group at a cysteine that is located in the α-chain sequence, only three amino acids distant from the glutamate that is responsible for normal C3 covalent fixation (12), attempts were made to elute C3b from C3b-ms by mild reduction with 20 mM cysteine. After this treatment, the binding of C3b-ms to human E was undiminished, suggesting that the C3b was primarily bound to microspheres by way of amino groups rather than by sulfhydral groups. This same mild reduction treatment of C3b-ATS completely eluted C3b that was disulfide bonded by way of the d region cysteine.

**DISCUSSION**

Assay of C-receptors with C3b- and C3d-coated fluorescent microspheres (C3b-ms and C3d-ms) had several advantages over other commonly used methods. Compared with other rosette assays with particles prepared with purified C components, only small amounts of C3b and C3d fragments, and no other purified C proteins, were required. C3-ms assays were more sensitive to cells with low numbers of receptors than other assays. Because C3-ms are relatively small and available for double-label assays with FITC surface staining. Finally, preliminary studies have indicated that fluorescent C3-ms may be useful for assay of cells in tissue sections (i.e., lymph node biopsy) and for analysis of C-receptor cells by fluorocytograph or fluorescence-activated cell sorter. Fluorescent microspheres coated with monoclonal antibodies to surface antigens have also been used successfully for analysis by fluorescence-activated cell sorter (21).

By using the procedures described, nonspecific binding of C3-ms to cells lacking C-receptors appeared to be minimal with all cell types examined. With C3b-ms, it was important to protect the bound C3b on the reagent from proteolysis at all stages. In particular, B cells, monocytes, and neutrophils have been shown to secrete endogenous enzymes, including I and

**TABLE II**

<table>
<thead>
<tr>
<th>C-Receptor Cell Type</th>
<th>Inhibition of Rosette Formation</th>
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<tr>
<td></td>
<td>C3b-ms</td>
</tr>
<tr>
<td></td>
<td>Anti-CR1</td>
</tr>
<tr>
<td>Human E</td>
<td>100  0</td>
</tr>
<tr>
<td>Blood lymphocytes</td>
<td>100  0</td>
</tr>
<tr>
<td>Blood monocytes</td>
<td>100  0</td>
</tr>
<tr>
<td>Lymphoblastoid lines</td>
<td>100  0</td>
</tr>
<tr>
<td>Daudi</td>
<td>ND  ND</td>
</tr>
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</table>

* ND—not done because untreated cells did not rosette.
elastase, that may convert C3b-ms to C3b-ms or C3d-ms and generate rosette formation with cells expressing CR2 and CR3, but lacking CR1, (15, 19, 20).

C3b and C3d fragments were prepared with trypsin on ATS and then eluted with cysteine (12). Because only proteins with a free sulfhydryl group bind to ATS, most minor contaminants in C3 preparations did not bind to ATS. Thus, partially purified C3 can be used for preparation of C3b-ms. Because the C3b is disulfide bonded to the ATS, the C3b-ATS generated with impure C3 can be washed with buffers containing high salt and nonionic detergents to elute noncovalently bound protein contaminants. After cleavage of C3b-ATS to C3d-ATS with trypsin, all C3c and smaller C3 fragments are washed away from the C3d-ATS before C3d elution with cysteine. Thus, column chromatography is not required to prepare pure C3b and C3d fragments from relatively impure C3 preparations.

Attempts were also made to coat fluorescent microspheres with F(ab')2-anti-CR1, and F(ab')2-anti-CR3. However, apparently because these antibodies were of relatively low titer, insufficient specific antibody was bound to the microspheres to any type of C-receptor-bearing cell. Attempts are now being made to prepare monoclonal anti-CR1, and anti-CR3 that may be useful for the microsphere assay.

REFERENCES